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 Transmittal Letter to the United States
 Designated/Elected Office (DO/EO/US)

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Attorney's Docket No. : A20-017
 U.S. Application No. : Not yet assigned
 International Application No. : PCT/AU99/00659
 International Filing Date. : 13 August 1999 (13.08.99)
 Priority Date Claimed : 13 August 1998 (13.08.98)
 Title of Invention : Monoclonal Antibody Inhibitor of GM-CSF, IL-3 and IL-5 and Other Cytokines and Uses Thereof
 Applicant(s) for (DO/EO/US) : Angel, LOPEZ and Richard D'ANDREA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. X This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. This express request to begin national examination procedures [35 U.S.C. 371 (f)] at any time rather than delay examination until the expiration of the applicable time limit set forth in 35 U.S.C 371(b) and PCT Articles 22 and 39(1).
4. X A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. X A copy of the International Application as filed [35 U.S.C. 371(c) (2)]
 - a) X is transmitted herewith (required only if not transmitted by the International Bureau)
 - b) has been transmitted by the international Bureau
 - c) is not required, as the application was filed in the United States Receiving Office (RO/US)
6. A translation of the International Application into English [35 U.S.C.371(c) (2)]
7. Amendments to the claims of the International Application under PCT Article 19 [35 U.S.C.371(c) (3)]
 - a) are transmitted herewith (required only if not transmitted by the International Bureau)
 - b) have been transmitted by the International Bureau
 - c) have not been made; however, the time limit for making such amendments has **NOT** expired.
 - d) have not been made and will not be made
8. A translation of the amendments to the claims under PCT Article 19 [35 U.S.C.371(c) (3)]
9. X An oath or declaration of the inventor(s) [35 U.S.C.371(c) (4)]
10. A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 [35 U.S.C.371(c) (5)]

Items 11. to 16. below concern other document(s) or information included:

11. X An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98
12. X An Assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
13. A **FIRST** preliminary amendment
- A **SECOND** or **SUBSEQUENT** preliminary amendment
14. A substitute specification
15. A change of power of attorney and/or address letter
16. X (other items or information) Form PCT/RO/101, Form PCT/RO/308, Form PCT/IB/332, PCT Published Application No. **WO 97/28190**, PCT Demand; Form PCT/IPEA/416, and Form PCT/IPEA/409.

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EXPRESS MAIL No.: **EL 704 550 135 US** Deposited: **February 13, 2001**

I hereby certify that this correspondence is being deposited with the United States Postal Service Express mail under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, DC 20231

Helen Tam

February 13, 2001
 Date

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17. X The following fees are submitted:

	CALCUL- ATIONS	PTO USE ONLY
BASIC NATIONAL FEE [37 CFR 1.492(a)(1)-(5)]:		
Search Report has been prepared by the EPO or JPO \$ 860.00		
International preliminary examination fee paid to USPTO [37 CFR 1.482] \$ 690.00		
No International preliminary examination fee paid to USPTO [37 CFR 1.482] but International search fee paid to USPTO [37 CFR 1.445(a)(2)] \$ 710.00		
<u>X</u> Neither International preliminary examination fee [37 CFR 1.482] nor International search fee [37 CFR 1.445(a)(2)] paid to USPTO \$ 1000.00	\$1000.00	
International preliminary examination fee paid to USPTO [37 CFR 1.482] and all claims satisfied provisions of PCT Article 33(1)-(4) \$ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT		\$1000.00
Surcharge of \$130.00 for furnishing the oath or declaration later than <u>20</u> 30 months from the earliest claimed priority date [37 CFR 1.492(e)]		
Claims	Number filed	Number extra
Total Claims	31 -20= 11	x \$ 18. =
Indep. Claims	5 - 3= 2	x \$ 80. =
Multiple Dependent Claim(s) (if applicable) + \$ 260. =		\$
TOTAL OF ABOVE CALCULATIONS =		\$1270.00
Reduction by 1/2 for filing by small entity, if applicable.		
Applicant claims small entity status. See 37 CFR 1.27 [Note 37 CFR 1.9, 1.27, 1.28]		
		\$ 635.00
SUBTOTAL =		\$ 635.00
Processing fee of \$130.00 for furnishing the English Translation later than <u>20</u> 30 months from the earliest claimed priority date [37 CFR 1.492(f)]		
TOTAL NATIONAL FEE =		\$
Fee for recording the enclosed assignment [37 CFR 1.21(h)]		
The assignment must be accompanied by an appropriate cover sheet [37 CFR 3.28, 3.31]. \$40.00 per property		
TOTAL FEES ENCLOSED =		\$ 675.00
(AMOUNTS TO BE REFUNDED OR CHARGED)		REFUNDED \$
		CHARGED \$

a) ✓ A check in the amount of **\$675.00** to cover the above fees is enclosed.

b) - Please charge my Deposit Account No. **04-0838** in the amount of \$
to cover the above fees. A duplicate copy of this sheet is enclosed.

c) ✓ The Commissioner is hereby authorized to charge any additional fees which
may be required, or credit any overpayment to Deposit Account No. **04-0838**.
A duplicate copy of this sheet is enclosed.

d) ✓ Applicant claims small entity status. See 37 CFR 1.27

NOTE: Where an appropriate time limit under 36 CFR 1.494 or 1.495 has not been met,
a petition to revive [37 CFR 1.137(a) or (b)] must be filed and granted to restore
the application to pending status.

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32,559

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February 13, 2001

Date

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MONOCLONAL ANTIBODY INHIBITOR OF GM-CSF, IL-3, IL-5 AND OTHER CYTOKINES, AND USES THEREOF

FIELD OF THE INVENTION

5 This invention relates to a method of isolating monoclonal antibody inhibitors and reagents derived therefrom and other inhibitors of cytokine binding including monoclonal antibodies and reagents derived therefrom and small molecules capable of inhibiting binding of GM-CSF, IL-3 and IL-5 to the common beta receptor subunit.

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INTRODUCTION

Human interleukin (IL)-5, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are cytokines involved in hemopoiesis and inflammation (Metcalf; 1986). All three cytokines stimulate eosinophil production, function and survival (Metcalf; 1986) and therefore have the ability to influence inflammatory diseases such as asthma, atopic dermatitis and allergic rhinitis where the eosinophil plays a major effector role. IL-5, being the eosinophil specific cytokine, has received most of the initial attention with IL-5 mRNA and protein levels noted to be elevated in lung tissue and bronchoalveolar lavage (BAL) fluid from symptomatic asthma patients (Fukuda *et al* 1994). Correlation between IL-5 levels and allergen challenge and disease activity have also been seen (Sur *et al*, 1996). It is becoming apparent, however, that not only IL-5 but also GM-CSF and IL-3 play a role in eosinophil production and activation in asthma as there is evidence of both GM-CSF and IL-3 being synthesized at sites of allergic inflammation (Bagley *et al*, 1997b; Allen *et al* 1997). It is possible that expression of these cytokines contributes to the total number of infiltrating eosinophils and the degree of eosinophil activation. Alternatively, they may be responsible for different phases of eosinophil infiltration. Recent kinetic data from patients undergoing antigen challenge showed that IL-5 levels increased between days 2-7 post challenge, whilst GM-CSF peaked at day 2, and remained elevated throughout day 16. Furthermore, GM-CSF detection extended beyond the site of allergen challenge.

IL-5, GM-CSF and IL-3 stimulate eosinophils and other normal and cancer cells by binding to cell surface receptors that comprise a ligand-specific α chain and a β chain which is shared by the three receptors (β_c) (Bagley *et al* 1997a). Binding to each receptor α chain is the initial step in receptor activation, however, engagement of either α chain alone is not sufficient for activation to occur. Recruitment of β_c

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by each ligand: α chain complex follows, a step that has two major functional consequences: firstly, it allows the binding of IL-5, GM-CSF and IL-3 to become essentially irreversible; and secondly, it leads to full receptor activation (Bagley *et al* 1997a). Since β_c is the major signalling component of these receptors its engagement leads to the activation of JAK-2, STAT-5 and other signalling molecules culminating in the full plethora of cellular activities commonly associated with either IL-5, GM-CSF and IL-3 stimulation such as eosinophil adherence, priming for degranulation and cytotoxicity, and prolongation of viability (Bates *et al*, 1996).

In order to block or antagonize the activity of eosinophil-activating cytokines *in vivo* three major approaches are being tried. One of them utilizes antibodies to the implicated cytokines. For example, antibodies to IL-5 are being used in an animal model of allergen-induced asthma and have shown to have a relatively long lasting effect in preventing eosinophil influx into the airways and bronchial hyperresponsiveness (Mausser *et al*, 1995). A second approach relies on IL-5 or GM-CSF mutants which can bind to the respective α chains with wild type affinity but which have lost or shown reduced ability to interact with human β_c . IL-5 mutants such as E13Q, E13K and E13R, and the human GM-CSF mutant E21R directly antagonize the functional activation of eosinophils by IL-5 or GM-CSF respectively (Tavernier *et al* 1995; McKinnon *et al* 1997; Hercus *et al* 1994b). However, at least in the case of E13K, eosinophil survival is not antagonized and in fact this mutant is able to support eosinophil survival (McKinnon *et al* 1997). A third approach involves the use of soluble receptor α chains which can sequester circulating cytokines. However, this carries the risk of a cytokine:receptor α chain complex potentially interacting with surface-expressed β_c and triggering receptor activation. The common theme amongst these approaches is that they tackle a single receptor system involving either IL-5, GM-CSF or IL-3 leaving the other two eosinophil-acting cytokines unaffected. Although the concomitant administration of IL-5 and GM-CSF antagonists may be considered, this may be clinically impracticable.

An alternative approach to blocking eosinophil-activating cytokines involves targeting the common β chain of their receptors. Although β_c does not directly bind IL-5, GM-CSF or IL-3 alone, it does bind to these cytokines complexed to the appropriate receptor α chain. Lopez *et al* in WO 97/28190, which is incorporated herein by reference in its entirety, have identified the major binding

sites of, β_c for the IL-5:IL-5R α , GM-CSF:GM-CSFR α and IL-3:IL-3R α complexes. Significantly, these sites are utilized by all three complexes and comprise the predicted B'-C' loop and F'-G' loop in β_c . Thus targeting β_c is not only desirable but also feasible, with the added potential to allow the simultaneous inhibition of IL-5, GM-CSF and IL-3 action by a single agent. These workers have shown that certain mutants in the B'-C' and the F'-G' loop fail to bind IL-5, GM-CSF and IL-3.

SUMMARY OF THE INVENTION

The present invention results from the isolation of a monoclonal antibody (BION-1) raised against the membrane proximal domain (domain 4) of β_c which is able to block the production and activation of human eosinophils stimulated by IL-5, GM-CSF or IL-3 and blocks the growth of leukaemic cell lines. This MoAb was able to block the high affinity binding of all three cytokines to eosinophils by binding to residues in the predicted B'-C' and F'-G' loops of β_c , and prevented receptor dimerization and β_c phosphorylation. It was found that raising an antibody capable of blocking the binding of all three cytokines was possible by screening monoclonal antibody-expressing hybridoma cell lines arising from immunising mice with cells expressing only domain 4 of β_c and lacking domains 1 to 3 and expressing domain 4 and the transmembrane and cytoplasmic regions.

Additionally this finding is likely to have implications for other members of the cytokine receptor superfamily some of which are shared subunits in a given subfamily (that is they bind several cytokines), and some which are ligand specific and bind to only one cytokine. The receptor α -chains for GM-CSF, IL-3 and IL-5 and β_c belong to the rapidly expanding cytokine receptor superfamily. Within this superfamily several sub-families are now emerging that are characterized by the sharing of a communal receptor subunit by multiple ligands: gp130 acts as an affinity converter and signal transducer for IL-6 (Hibi *et al.*, 1990; Taga *et al.*, 1992), IL-11 (Hilton *et al.*, 1994), oncostatin M (Liu *et al.*, 1992), ciliary neurotrophic factor, leukaemia inhibitory factor (LIF) (Ip *et al.*, 1992) and cardiotrophin-1 (Pennica *et al.*, 1995); the LIF receptor (LIFR) also binds ciliary neurotrophic factor (Davis *et al.*, 1993), cardiotrophin-1 (Pennica *et al.*, 1995) and oncostatin M in addition to LIF (Gearing *et al.*, 1994); IL-2R β supports affinity conversion and signalling of IL-2 and IL-15 (Giri *et al.*, 1994); IL-2R γ chain affinity converts IL-2 (Takeshita *et al.*, 1992), IL-4 (Russell *et al.*, 1993), IL-7 (Noguchi *et al.*, 1993), IL-9 (Kimura *et al.*, 1995) and IL-15 (Giri *et al.*, 1994);

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- evidence also suggests that IL-4 and IL-13 share a receptor component (Zurawski *et al.*, 1993) and this subunit has recently been cloned (Hilton *et al.*, 1996). It is not known which residues in gp130, LIFR and IL-2R β and γ chains are important for ligand binding or indeed whether different ligands share or have unique sets of binding determinants on these communal receptor subunits. Because these common subunits are vital for transducing signals by several ligands, the possibility arises that interfering with the ability of these common subunits to bind ligand or to form homodimers may affect the action of more than one ligand.
- 10 Clear similarities in structure between β_c and other cytokine receptors have been recognised and similarities in at least part of the binding site, the F-G' loop, have been identified in Lopez *et al.* in WO 97/28190. Accordingly it is an expectation that the means employed by the inventors to obtain a monoclonal antibody that inhibits binding of IL-3, GM-CSF and IL-5 will also lead to the isolation of
- 15 monoclonal antibodies that inhibit binding of other cytokines to their respective receptors.

- In a broad form of a first aspect the invention could be said to reside in a method of isolating a monoclonal antibody capable of inhibiting any one of IL-3, GM-CSF and IL-5 binding to the common receptor β_c , or a monoclonal antibody capable of inhibiting a cytokine binding to a receptor analogous to β_c , said method comprising the step of immunising an animal with a cytokine receptor or portion of a cytokine receptor containing the critical binding site which portion might include the extracellular domain 4 or analogous domain in the analogous common receptor
- 20 or part thereof, isolating antibody producing cells from said animal and fusing antibody producing cells with a myeloma cell line, screening for a cell line that produces an antibody of the desired type.

- The immunisation may involve introducing a cDNA clone of a portion of or all of the common receptor including the extracellular domain 4 or analogous domain in the analogous common receptor or part thereof, into a cell and proliferating said cells to form a recombinant cell line, inoculating an animal with said recombinant cell line, isolating antibody producing cells from said animal and fusing the antibody producing cell line with a myeloma cell line to form a hybridoma cell line,
- 30 screening for a hybridoma cell line that produces an antibody that binds to the recombinant cell line but not to the parent, and then testing for inhibition against all

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three cytokines. In one form the cell into which the cDNA clone is introduced is mammalian and one commonly used mammalian cell line is a COS cell.

The cDNA may encode a full or partial portion of domain 4 when it is in a configuration where the F'-G' loop and/or the B'-C' loop is in its native shape. The data below show that cDNA encoding substantially only domain 4 of the extracellular portion of β_c as well as the transmembrane and the intracellular portions maintains these sites in a sufficiently integral conformation so that an antibody raised thereagainst will give the inhibition sought. It is postulated that the same will be the case for analogous receptors for the cytokine superfamily. This method should be distinguished from immunising with the whole receptor since the extracellular domain 4 is likely to be covered or masked by other domains in the whole receptor.

β_c has two repeats of the cytokine receptor module (CRM), each of which has two discrete folding domains (CRDs), so that in total β_c has 4 domains hence named domains 1 to 4 ($\beta 1$ to 4). It is postulated that domain 2 of any CRM may be an equivalent of domain 4 and therefore domain 2 may be used in the immunisation.

In addition the domain 4 of β_c or equivalent domain in other cytokine receptors may be expressed in isolation in a microbial host such as *Escherichia coli* and used to immunise animals for developing monoclonal antibodies.

The analogous receptor may be any one of the cytokine superfamily receptors but not limited to the group comprising β_c , LIFR, gp130, IL-2R β , IL-4R/IL-13R, IL-2R γ , IL-3R α , EPOR, TPOR and OBR.

It will be understood that in one specific form of this aspect of the invention the method is used to isolate a monoclonal antibody that inhibits cytokine binding to a common receptor subunit. The common receptor is envisaged to be selected from the group of receptors acting for more than one cytokine including but not limited to gp130, LIFR, IL2R β /IL2R α , and IL-4R/IL-13R in addition to β_c .

It will also be understood that the invention encompasses monoclonal antibodies or fragments thereof produced as a result of this first form of the invention.

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In a broad form of a second aspect the invention could be said to reside in a monoclonal antibody, or fragments thereof capable of inhibiting the binding of the three cytokines IL-3, GM-CSF and IL-5 to the β_c receptor.

- 5 The degree of inhibition may range from complete inhibition to moderate inhibition, which inhibition will of course be dependent on the amount of monoclonal antibody or fragments thereof added to inhibit and the relative affinity of the antibody or fragment thereof to the β_c .
- 10 The extent of inhibition of respective ones of the three cytokines is not necessarily identical and may vary, so the different cytokines may be inhibited from binding to different degrees.

- The antibody fragments may be larger portions such as Fab fragments or much smaller fragments of the variable region. These fragments may be used as separate molecules or alternatively may form part of a recombinant molecule which is then used for therapeutic purposes. Thus for example the monoclonal antibody may be "humanised" by recombining nucleic acid encoding the variable region of the monoclonal antibody with nucleic acid encoding non-variable regions of human origin in an appropriate expression vector.
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- The inhibition preferably leads to blocking of at least one function of all three cytokines. One of the benefits that is proposed to be derived from these antibodies or antibody fragments is their use in modifying cells stimulated by one of the three cytokines, and more in one specific form modifying the activity of the three cytokines is proposed to impact greatly on eosinophil function. Therefore preferably the activity leads to inhibition of stimulation of effector cell activation and where the antibody or fragment thereof is to be used for treatment of asthma leads most preferably to inhibition of IL-5, IL-3 & GM-CSF mediated eosinophil activation. It will be understood however that cells other than eosinophils are also the effectors of adverse conditions in humans and animals as a result of stimulation by these cytokines and inhibition of such stimulation is also contemplated by this invention. These include cells that express either one or all of GM-CSF, IL-3 and IL-5 receptors, the stimulation of which leads to pathology. Examples of these are leukaemic cells, endothelial cells, breast cancer cells, prostate cancer cells, small cell lung carcinoma cells, colon cancer cells, macrophages in chronic inflammation
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such as rheumatoid arthritis, dendritic cells for immunosuppression and neutrophils in inflammation.

5 This in one form the invention may be said to reside in an inhibitor of leukaemic cell growth wherein the inhibitor is capable of inhibiting the binding of one or all of IL-3, GM-CSF and IL-5 to the β_c receptor. The inhibitor may be BION-1 or an agent capable of inhibiting BION-1 binding with β_c .

10 A number of different facets of eosinophil function might be modified so that in one form IL-5, IL-3 & GM-CSF mediated eosinophil survival is inhibited or blocked. In a second form IL-5, IL-3 and GM-CSF mediated eosinophil activation is inhibited or blocked.

15 In one form of this second aspect of the invention the monoclonal antibody or fragment thereof binds to at least the F'-G' loop of domain 4 of the β_c subunit.

20 In an alternative form the monoclonal antibody or fragment thereof binds to at least the B'-C' loop of domain 4 of the β_c subunit but this alternative form is not limited to monoclonal antibodies or fragments thereof that only bind to the F'-G' loop but includes monoclonal antibodies or fragments thereof that perhaps binds to both the F'-G' as well as the B'-C' loop of domain 4 of the β_c .

25 It is thought that the monoclonal antibody isolated by the inventors inhibits dimerisation of the common receptor units and thus the invention might encompass an antibody or fragments thereof of the second aspect of the invention that inhibit β_c receptor dimerisation.

30 In one very specific form the monoclonal antibody is the antibody produced by the hybridoma cell line BION-1 (ATCC HB-12525).

In a broad form of a third aspect the invention could be said to reside in a hybridoma cell line capable of producing a monoclonal antibody of any form of the first or second aspect of the invention.

35 In one specific form of the third aspect of the invention the hybridoma cell line is BION-1 (ATCC HB-12525).

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Since GM-CSF, IL-3 and IL-5 need to bind their respective α chains before being able to interact with β_c , at present most screening for new inhibitors utilise cell-based assays where both, α and β_c receptor units are co-expressed. Solid phase assays rely on inhibition of GM-CSF, IL-3 or IL-5 to their respective α chain only since these cytokines cannot bind to β_c alone. Since BION-1, unlike these three cytokines, can directly bind to β_c we propose that it can be used as a novel solid phase screening assay. Any compound that binds the appropriate site which is likely to inhibit all three cytokines will also inhibit the binding of BION-1. Additionally once further inhibitory compounds are uncovered these could be used in the place of BION-1 in that screening process. This therefore facilitates the screening of larger number of candidate inhibitor compounds.

In a broad form of a fourth aspect therefore the invention could be said to reside in a method of screening peptides, oligonucleotides and other small molecules for their capacity to competitively inhibit the binding of BION-1 or the binding of an agent capable of inhibiting BION-1 binding, to the β_c subunit.

Generally the screening assay involves contacting BION-1 or fragment thereof with the β_c subunit or fragment thereof as well as a candidate inhibitory compound, and measuring the degree of binding.

A reporting means is preferably provided to facilitate the detection of binding of BION-1 or fragment thereof with β_c subunit or fragment thereof. Thus, for example, a competitive binding assay using labelled BION-1 could be used for this purpose. β_c or domain 4 of β_c is immobilized on a plate or tube and several compounds added, followed by labelled or tagged BION-1 or fragments thereof. Since BION-1 binds the region of β_c involved in binding all three cytokines, any compounds that block or reduce the binding of BION-1 or fragments thereof to β_c or domain 4 will be considered candidate inhibitory compounds. Thus, the availability of BION-1 as an agent that for the first time allows the direct binding to the cytokine binding region of β_c affords a novel test for the identification of simultaneous inhibitors of GM-CSF, IL-3 and IL-5. It will be understood that the same will apply for other cytokines and their respective receptors.

It will be understood that not the entire β_c subunit needs be used to screen candidate compounds, and certainly the present data indicates that a fragment of the

β_c subunit encompassing domain 4 has sufficient structure in common with the native β_c subunit to reflect the configuration of the cellular target for an inhibitor useful for an *in vivo* effect.

- 5 In a broad form of a fifth aspect, the invention could be said to reside in a cytokine inhibitor capable of simultaneously blocking the binding of β_c by IL-3, GM-CSF, and IL-5 made according to the fourth aspect of the invention.

- 10 It is thought that compounds that inhibit binding of the IL-3, IL-5 and GM-CSF to the β_c will be therapeutically useful for intervention in conditions where IL-3, GM-CSF and IL-5 play a pathogenic role, mainly allergy, asthma, acute and chronic myeloid leukaemias, lymphoma and inflammation including rheumatoid arthritis, breast cancer and prostate cancer.

- 15 Similarly for other common cytokine receptors it is thought that antagonists or agonists will be therapeutically useful. gp130 is functionally analogous to β_c in that it is a common binding sub-unit and signal transducer for the IL-6, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF) and IL-11. It is suggested that raising an antibody against a domain analogous to domain 4 of β_c will also lead to blocking of two or more of these cytokines.

- 20 Antagonism of this receptor system will be useful in inflammation, leukaemia and lymphoma. Antagonists to IL2R β /IL2R α may be useful as immunosuppressants. An agonists of LIFR may be useful for the prevention of implantation of embryos in uteri. Antagonists of IL-4/IL-13 will inhibit IgE production and may be useful
25 in treating asthma and allergies.

BRIEF DESCRIPTION OF THE DRAWINGS

- 30 Figure 1. Flow cytometry analysis of the staining of MoAb BION-1 (continuous line) and an isotype matched IgG₁ control MoAb (dotted line) to (A) COS cells transiently transfected with β_c , (B) CHO cells constitutively expressing β_c , (C) TF-1.8 cells, (D) neutrophils, (E) eosinophils and (F) monocytes.

- 35 Figure 2. MoAb BION-1 recognizes β_c protein. (A) Immunoprecipitation of β_c from ¹²⁵I-surface labelled CHO β_c

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cell lysate. Both experiments were performed on 7.5% SDS PAGE under reducing conditions. (C) MoAb BION-1, but not another anti- β_c MoAb 1C1, recognises a β_c mutant (β_c - Δ QP) that contains only domain 4 in the extracellular regions by immunoprecipitation of CHO Δ QP cells. This mutant has a flag attached so it can also be seen by anti-flag MoAb M2. The experiment was performed on 10% SDS PAGE under reducing conditions.

Figure 3.

Dose-dependent competition for the binding of ^{125}I -IL-5 (50 pM), ^{125}I -GM-CSF (50 pM) and ^{125}I -IL-3 (200 pM) by MoAbs BION-1 (•), an anti- β_c MoAb control (), and an IgG₁ control MoAb (o) to TF-1.8 cells (2×10^6 per point). (---) represents ligand binding in the presence of 200-fold excess unlabelled ligand. Each point is the mean of triplicate determinations.

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Figure 4.

A Fab fragment of BION-1 blocks high affinity binding of IL-5, GM-CSF and IL-3. Binding of ^{125}I -IL-5 (50 pM), ^{125}I -GM-CSF (50 pM) and ^{125}I -IL-3 (200 pM) were assessed on TFI.8 cells (2×10^6 per point) in the presence or absence of 3000 nM MoAb BION-1, or control anti- βc MoAb, or 4200 nM Fab fragment of BION-1 for 2 h at room temperature. IC1 was used as a non blocking anti- βc control. Cells were separated from unbound radioligand by spinning through FCS and the resulting cell pellet was counted. The results are expressed as a percentage of the total specific cpm bound seen in the absence of antibody. Non-specific binding was determined in the presence of 200 fold excess of cold ligand. Total binding seen for ^{125}I -IL-5, ^{125}I -GM-CSF and ^{125}I -IL-3 were 9744.2, 2567.1 and 3379.13 cpm respectively. Blocking with Fab fragment of BION-1 was determined from a single point and BION-1 and IC1 values are the mean of duplicate determinations with error bars representing 1 standard deviation.

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Figure 5. BION-1 blocks high affinity binding of IL-5, GM-CSF and IL-3 to human eosinophils. Human eosinophils (1.8×10^6 per point) were incubated with ^{125}I -IL-5 (10 pM or 3 pM), ^{125}I -GM-CSF (50 pM) or ^{125}I -IL-3 (200 pM) either alone or in the presence of $1 \mu\text{M}$ MoAb at room temperature for 2 h. MoAb's 9E10 and 8E4 were used as isotype matched and non-blocking anti- β_c control respectively for BION-1. Cells were separated from unbound radioligand by spinning through FCS and the resulting cell pellet was counted. The results are expressed as a percentage of the total specific cpm bound seen in the absence of antibody. Non-specific binding was determined in the presence of 200 fold excess of cold ligand and was determined to be an average of 0.3% of total counts added. Total binding seen for ^{125}I -IL-5, ^{125}I -GM-CSF and ^{125}I -IL-3 were 765, 622 and 748 cpm respectively. Each point is the mean of duplicate determinations and error bars represent 1 standard deviation.

Figure 6. MoAb BION-1 recognizes an epitope in β_c comprising at least residues M363, R364, E366 and R418. Human β_c wild type and mutants (Woodcock *et al*, 1996) were tested for reactivity with MoAb BION-1 and 1C1 used as a control. Following expression of wild type β_c and β_c mutants on COS cells, β_c was immunoprecipitated by MoAb 8E4, followed by Western blotting by MoAb BION-1 (top) or the non blocking MoAb 1C1 (bottom).

Figure 7. The binding of ^{125}I -labelled MoAb BION-1 (1 nM) to TF.1 cells is inhibited by IL-3 (\bullet), but not by $\text{TNF-}\alpha$ (\circ). (---) Represents inhibition in the presence of 200-fold excess of unlabelled BION-1.

Figure 8. BION-1 IgG selectively inhibits IL-5, GM-CSF and IL-3 mediated proliferation of TF 1.8 cells. The proliferation experiments represent the comparison of inhibition of BION-1 IgG at maximal dosage (400 $\mu\text{g/ml}$ BION-1 with IL-5 and IL-3 and 850 $\mu\text{g/ml}$ with GM-CSF) against ED_{50}

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concentrations for IL-5 (0.3 ng/ml), GM-CSF (0.03 ng/ml) or IL-3 (0.3 ng/ml). An anti- β_c antibody and an irrelevant IgG antibody were used as controls. The results are expressed as DPM. Each value represents the mean of triplicate determinations and error bars represent the SEM.

Figure 9. Fab fragment of BION-1 and BION-1 IgG inhibits IL-5, GM-CSF and IL-3 mediated proliferation of TF1.8 cells. Intact IgG or Fab fragment of BION-1 were titrated against a fixed concentration of IL-5 (0.3 ng/ml), GM-CSF (0.03 ng/ml) or IL-3 (0.3 ng/ml) in proliferation assays where TF1.8 cells at 5×10^4 /well were incubated for 48 hours and then pulsed for 5 hours with 0.5 μ Ci/well 3 H-Thymidine. The results are expressed as DPM. Each value represents the mean of triplicate determinations and error bars represent the SEM.

Figure 10. Eosinophil survival. (A) Viability of eosinophil after 36 hours in the presence of IL-5, IL-3 and GM-CSF. (B) Viability of eosinophil after 36 hours in the presence of IL-5, IL-3 and GM-CSF (1 nM) and different concentrations of MoAb BION-1 (o) and 8E4 (*). Each point is the mean of triplicate determinations from three samples and error bars represent 1 standard deviation.

Figure 11. MoAb BION-1 inhibits IL-5-stimulated CD69 up-regulation on human eosinophils. (A) CD69 up-regulation in the presence of different concentrations of IL-5, IL-3, GM-CSF and TNF- α . (B) CD69 up-regulation stimulated by 1 nM of IL-5, GM-CSF, IL-3 or TNF- α in the presence of different concentrations of MoAb BION-1 or control anti- β_c MoAb 8E4. Each point is the mean value of three replicates and error bars represent 1 standard deviation.

Figure 12. Inhibition of IL-3-induced α and β chain dimerization and phosphorylation by MoAb BION-1. Immunoprecipitations using anti-IL-3R α MoAb 9F5 or anti- β_c MoAb 8E4 from

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M07e cells preincubated with MoAbs BION-1, MoAb 1C1 or medium alone (-) for 1 min, before being stimulated (+) or not (-) with IL-3 (50 nM) for 5 min. The figure was visualised by PhosphorImaging and the position and molecular weight (in thousands) of marker proteins are shown to the left of the gels. The gels were reprobed by Western blotting analysis using anti-phosphotyrosine MoAb 3-365-10 and the top panel shows the image of part of the gels in the β_c area.

Figure 13. Screening peptides for inhibition of MoAb BION-1 binding to soluble β_c domain 4 adsorbed to solid phase. *E. coli* derived soluble β_c domain 4 ($s\beta_c\#4$) was coupled to Maxisorp ELISA plates at 10 μ g/ml in 0.1M carbonate buffer overnight and then blocked with 1% BSA. (A) B45.pep (FHWWWQP-GGGCDYDDDK) (+) and (B) YB12.pep (FPFWYHAHSPWS-GGGCDYKDDDK) (•) were derived from biopanning libraries with $s\beta_c\#4$ using an acid eluant. B45 was allowed to bind to $s\beta_c\#4$ at 0.0125 μ M and YB12 was allowed to bind $s\beta_c\#4$ at 0.025 μ M. BION-1 was added to the plates at a starting concentration of 5 μ g/ml and serial dilutions were used to titrate the BION-1 down to 0.004 μ g/ml. The plate was washed again and BION-1 binding to $s\beta_c\#4$ was detected.

Figure 14. BION-1 specifically inhibits the growth *in vitro* of chronic myelomonocytic cells (CMML). A control antibody (1C1) does not inhibit.

DETAILED DESCRIPTION OF THE INVENTION

Materials and Methods

ΔQP cDNA: To express domain 4 of β_c on the cell surface we cloned the activated β_c mutant, $h\beta_c\Delta QP$, with an extracellular deletion removing domains 1 to 3

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(D'Andrea *et al* 1996), into the eukaryotic expression vector pcDNA3 (Invitrogen).

Cytokines and cell lines: Recombinant human IL-3 and GM-CSF were produced in *E. coli* as described (Barry *et al* 1994, Hercus *et al* 1994b). Recombinant human IL-5 was purified from *E. coli* by Bresatec (Adelaide, South Australia). Recombinant EPO was purchased from Johnson & Johnson (New Jersey). TNF α was a gift from Dr. J. Gamble in the Hanson Centre for Cancer Research. COS cells were transfected with receptor cDNA as described previously (Woodcock *et al* 1994). CHO β_c and CHO Δ QP cells stably expressing either full length β_c or domain 4 respectively were generated by electroporation (Hercus *et al*, 1994a). TF1.8 cells were a gift from Dr J. Tavernier from University of Gent, Belgium. MO7e cells, a human megakaryoblastic cell line, were from Dr P Crozier, Auckland, New Zealand. Human eosinophils were purified from the peripheral blood of slightly eosinophilic volunteers via sedimentation through dextran and centrifugation through a discontinuous density gradient of hypertonic Metrizamide, as previously described (Vadas *et al* 1979). Eosinophils were more than 92% pure. Human neutrophils and monocytes were purified from peripheral blood as described previously (Lopez *et al*, 1990) with more than 95% purity.

Generation of anti- β_c MoAbs: BALB/c mice were immunized intraperitoneally with 1×10^7 COS cells transfected with β_c or Δ QP expression constructs. Δ QP constructs express substantially only domain 4 of the extracellular domains of β_c . The immunizations were repeated 4 times at two-weekly intervals. Four weeks after the final immunization, a mouse was boosted with 2×10^6 COS transfectants intravenously. Three days later, splenocytes were harvested and fused with NS-1 myeloma cells as previously described (Sun *et al* 1996). Hybridoma supernatants were screened on CHO β_c or CHO Δ QP cells by flow cytometry, with untransfected CHO cells as a control. All antibodies were from single hybridoma clones as selected by limiting dilution method. MoAbs were purified from ascites fluid or hybridoma supernatant by a protein A sepharose column. The isotypes of MoAbs were tested with a Mouse MoAb Isotyping Kit (Boehringer Mannheim, Germany). Fab fragments were generated using a Fab Preparation kit (Pierce, Rockford, IL) following the supplied protocol.

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Immunofluorescence: Freshly purified neutrophils, eosinophils, monocytes, or CHO and COS cell transfectants (5×10^5) were incubated with 50 μ l of hybridoma supernatant or 0.25 mg of purified MoAb for 45-60 min at 4°C. Cells were washed twice and then incubated with FITC-conjugated rabbit anti-mouse Ig (Silenus, Hawthorn, Victoria, Australia) for another 30-45 min. Cells were then washed and fixed before analysing their fluorescence intensity on an EPICS-Profile II Flow Cytometer (Coulter Electronics). Two colour staining was carried out by additional incubation with another MoAb directly coupled to PK.

Ligand Binding Assay: IL-3 and GM-CSF were radio-iodinated by the iodine monochloride method (Contreras 1983). 125 I-IL-5 was purchased from Dupont NEN (North Sydney, NSW, Australia). Binding assays were performed as previously described (Lopez *et al* 1989). Briefly, $1-2 \times 10^6$ TF-1.8 cells were preincubated with BION-1 Fab fragments, anti- β_c or control MoAbs over a concentration range of 0.06 to 4200 nM for 1 hour. Radio-labelled ligand was then added and incubated for a further two hours before the cells were separated from free label by spinning through FCS. Counts associated with the resulting cell pellets were determined by counting on a γ counter (Cobra Auto Gamma; Packard Instruments Co, Meriden, CT). Non-specific binding was determined for each ligand by binding in the presence of a 200 fold excess of unlabelled cytokine.

MoAb binding assay: MoAbs were radio-iodinated by the chloramine-T method (McConahey 1980). Saturation binding studies were performed by incubating 2×10^6 TF1 cells in a range of concentrations of radio-labelled antibodies in the presence or absence of excess unlabelled antibodies. The binding affinity of each anti- β_c MoAb to its antigen was determined by Scatchard transformation (Scatchard 1949) and analysed with the ligand program (Munson and Rodbard, 1980). Competition binding experiments were set up by preincubating the TF1.8 cells with a range concentration of IL-3, or GM-CSF, or IL-5 prior to adding radio-labelled MoAb QP1 for two hours as per ligand binding assay. Epitope analysis was determined by testing the capacity of each unlabelled MoAb to compete for the binding of each radio-labelled MoAb to the β_c on COS cell transfectant.

Co-immunoprecipitation of α and β chains and the β_c phosphorylation assays: M07 cells were surface labelled with 125 I by the lactoperoxidase method as

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described previously (Walsh and Crumpton, 1977). The labelled cells incubated in either medium containing IL-3 (100 ng/ml) alone or IL-3 together with the MoAb QP1, 1C1 (0.5 mg/ml) or 7G3 (30 mg/ml) for 5 min. Cells were lysed in lysis buffer consisting of 137 mM NaCl, 10 mM Tris-HCl (pH 7.4), 10% Glycerol, 1 % Nonidet P40 with protease and phosphatase inhibitors (10 mg/ml leupeptin, 2mM phenylmethanesulphonyl fluoride, 10 mg/ml aprotinin and 2 mM sodium vanadate) for 30 min at 4°C followed by centrifugation of the lysate at 10,000 x g for 15 min to remove cellular debris. The lysate was precleared with mouse-Ig-coupled Sepharose beads for 18 h at 4°C and incubated with anti IL-3Ra, anti- β_c MoAb beads for 2 hr at 4°C. The beads were washed 6 times with lysis buffer and immunoprecipitated proteins were separated by SDS-PAGE under reducing condition. The immunoprecipitated proteins were detected by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The gels were then reprobed by Western blotting analysis with an anti-phosphotyrosine MoAb, 3-365-10 (Boehringer Mannheim, Frankfurt, Germany).

TF-1.8 cell proliferation assay: TF-1.8 cells were grown in the presence of 2 ng/ml of GM-CSF. The cells were starved for 24 hours before setting up proliferation assays as described previously (Sun *et al.*, 1996). From dose-response curves the half-maximal proliferation dosage of IL-3 (0.3 ng/ml), GM-CSF (0.03 ng/ml), IL-5 (0.3 ng/ml) or EPO (5 ng/ml) was chosen to perform proliferation experiments in the presence of a range of concentrations of MoAbs. The ³H-Thymidine incorporation of each sample was determined by liquid scintillation and expressed as disintegrations per minute (DPM).

Eosinophil survival assays: The maximal dose of IL-5 required to support eosinophil survival after 36 hours was determined. Eosinophils were then cultured with 1 nM of IL 5 plus anti- β_c MoAbs for 36 hours. The viability of eosinophils was quantitated by propidium iodide staining and flow cytometry analysis as described (Nicoletti, 1991).

CD69 expression: CD69 expression on eosinophils was measured by means of an anti CD69 monoclonal MoAb coupled to PE by flow cytometry.

β_c mutants and MoAb Mapping: Single amino acid substitutions in the B'-C' and F'- 3' loops of domain 4 of the β_c have been described previously (Woodcock, *et*

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al., 1994; 1996). The cDNAs for wild type β_c and each of the β_c mutants in the B'-C' and F'-G' loops were introduced into COS cells by the electroporation (Hercus *et al.*, 1994). Cell transfectants were analysed for surface expression with 48 hours after transfection. Mutants on the B' and C' β -strands such as

5 L3: 6N, W358N, I374N and Y376N were expressed on FDCP1 cells from retroviral expression constructs (Jenkins *et al.*, 1995). Epitope-mapping of anti- β_c antibodies was analysed by Immunofluorescent study. The anti- β_c MoAbs were tested for their abilities to recognise wild type β_c and the β_c mutants analysed by flow cytometer using standard immunofluorescence method. For each mutant, the

10 experiment was repeated at least twice.

BION-1 binding inhibitory peptides: *E. coli* derived soluble β_c domain 4 ($s\beta_c\#4$) was coupled to Maxisorp ELISA plates at 10 μ g/ml in 0.1M carbonate buffer overnight and then blocked with 1% BSA. B45.pep (FHWWWQP-

15 GGGCDYDDDK) was derived from four rounds of biopanning the Ph.D-7mer library with $s\beta_c\#4$ using an acid eluant. YB12.pep (FPFWYHAHSPWS-GGGCDYKDDDK) was derived from biopanning the Ph.D-12mer library with $s\beta_c\#4$ using an acid eluant. B45 was allowed to bind to $s\beta_c\#4$ at 0.0125 μ M and YB12 was allowed to bind $s\beta_c\#4$ at 0.025 μ M. The plate was washed in TBS +

20 0.5 % Tween. BION-1 was added to the plates at a starting concentration of 5 μ g/ml and serial dilutions were used to titrate the BION-1 down to 0.004 μ g/ml. The plate was washed again and BION-1 binding to $s\beta_c\#4$ was detected with α -mouse conjugated to HRP, using a colour based reaction which was read on a plate counter by absorption.

25 *BION-1 inhibition of chronic myelomonocytic cells:* Peripheral blood from a patient with chronic myelomonocytic leukemia was centrifuged over Ficoll-Paque to separate the mononuclear cells. After washing and counting, the cells were plated on agar as a concentration of 10⁵ per plate. After incubation in medium

30 containing monoclonal antibodies BION-1 or 1C1, with or without IL-3, for 14 days at 37°C the number of arising colonies were counted by microscopical examination. Each cell cluster containing more than 40 cells was counted as a colony.

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Results

Development of MoAb BION-1

Previous experiments have shown that the putative F'-G' loop of β_c contains a common binding site for IL-5, GM-CSF and IL-3 (Woodcock *et al.* 1996; WO 97/28190). We have now produced a blocking compound, represented by MoAb BION-1, by immunizing mice with COS cells transfected with a cDNA coding for domain 4 of β_c . Screening of hybridoma supernatants was performed on a CHO cell line expressing domain 4 of β_c . One hybridoma cell line was identified which produced a MoAb which specifically recognized this cell line and not a parental CHO cell line not expressing domain 4 of β_c . This MoAb was termed BION-1 and was characterized in biochemical, binding and biological experiments.

BION-1 recognizes domain 4 as well as wild type β_c .

MoAb BION-1 was tested for reactivity against cell lines transfected with β_c and against primary cells known to express IL-5, GM-CSF and IL-3 receptors. BION-1 recognized COS cells transiently transfected with β_c , CHO cells permanently transfected with β_c , the erythroleukaemic TF-1 cell line, and purified peripheral blood human neutrophils, eosinophils and monocytes (Figure 1).

The antigen recognized by BION-1 was confirmed to be domain 4 of β_c by biochemical analysis of transfected cells. Figure 2A shows that BION-1 immunoprecipitated a surface 125 I-labelled protein of about 120,000 MW consistent with the size of β_c . Similarly, BION-1 recognized a protein of 120,000 MW by Western blotting using lysates of CHO cells expressing full length wild type β_c (Figure 2B). The size of these bands also corresponded to the bands recognized by a previously developed anti- β_c MoAb (Korpelainen *et al.* 1993; Woodcock *et al.* 1996). To formally show that BION-1 recognized domain 4 of β_c we also tested BION-1 for its ability to immunoprecipitate domain 4 expressed on the surface of CHO cells. As a positive control we incorporated a short polypeptide to the N-terminus of domain 4 (flag epitope) to which a MoAb has been previously developed. As a negative control, we used the anti- β_c MoAb 1C1 which recognizes an epitope located elsewhere in β_c . Figure 2C shows that BION-1 immunoprecipitated a band of about 80,000 MW from 125 I-surface labelled-domain 4-expressing CHO cells consistent with the expected size of domain 4. MoAb M2 against the flag epitope added to domain 4 of β_c also precipitated a similar size protein. In contrast, MoAb 1C1 failed to immunoprecipitate domain 4. These

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experiments show that BION-1 can specifically recognize domain 4 of β_c on the surface of cells and following denaturation of the protein.

BION-1 inhibits the high affinity binding of IL-5, GM-CSF and IL-3 to TF-1 cells and to human eosinophils

Given that domain 4 of β_c is crucial for the high affinity binding of IL-5, GM-CSF and IL-3, we examined whether BION-1 was able to affect this binding. We found that BION-1 inhibited in a dose-dependent manner the binding of ^{125}I -IL-5, ^{125}I -GM-CSF and ^{125}I -IL-3 to the human erythroleukaemic cell line TF-1. For each radioligand we used the smallest possible concentration to maximize the possibility of measuring high affinity. This can be more readily achieved with IL-3 and GM-CSF for which the difference between the low affinity component (provided by each α chain alone) and the high affinity component (provided by co-expressing β_c with each α chain) is about 1,000 fold and 30 fold respectively. In the case of IL-5, the affinity conversion of β_c is only in the 25 fold range, hence, high and low affinity binding cannot be clearly separated. This is likely to explain why BION-1 shows complete inhibition of ^{125}I -GM-CSF and ^{125}I -IL-3 binding (Figure 3). The residual ^{125}I -IL-5 binding seen with high concentrations of BION-1 is likely to be the result of low affinity ^{125}I -IL-5 binding (α chain) which BION-1 would not be expected to inhibit. This is consistent with BION-1 inhibition of ^{125}I -IL-5 binding reaching a plateau beyond which no further inhibition can be detected (Fig 3a). Other anti- β_c MoAb (anti- β_c control) and the IgG₁ MoAb control did not inhibit ^{125}I -IL-5, ^{125}I -GM-CSF and ^{125}I -IL-3 binding to TF-1 cells (Figure 3).

The blocking effect of BION-1 was seen whether the MoAb was used as purified IgG or as Fab' fragment. Figure 4 shows that the Fab' fragment of BION-1 blocked the binding of 50 pM ^{125}I -IL-5, 50 pM ^{125}I -GM-CSF and 200 pM ^{125}I -IL-3 to TF-1 cells.

Since one of the major clinical utilities of blocking IL-5, GM-CSF and IL-3 binding is likely to be in asthma, a disease in which eosinophils are believed to play a major role, it was important to test whether BION-1 could block the binding of IL-5, GM-CSF and IL-3 to these cells. As shown in Figure 5, BION-1 inhibited the binding of all three radio-labelled cytokines to purified human

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eosinophils. In contrast, other anti- β_c MoAb or the IgG₁ MoAb control failed to do so.

Epitope mapping of BION-1

- 5 The fact that BION-1 inhibited the binding of 125 I-IL-5, 125 I-GM-CSF and 125 I-IL-3 to TF-1 cells and eosinophils suggested that it might be binding to the critical region in β_c to which these cytokines bind or at least in close proximity to it. To try to define the region/epitope in β_c recognized by BION-1, we used several mutants of β_c and examined whether substitutions of individual amino acids in the
- 10 predicted B'-C' loop or F'-G' loop impaired BION-1 binding. Two sets of experiments were carried out. In the first instance we immunoprecipitated wild type β_c from transfected COS cells with a MoAb anti- β_c . The immunoprecipitates were then tested for reactivity with the control anti- β_c MoAb 1C1, or BION-1. The results shows that β_c mutants carrying the substitutions M363A/R364A, or
- 15 E366A or R418A were not recognized by BION-1 (Figure 6). In a second set of experiments, the direct binding of radio-labelled BION-1 was measured on transfectants expressing the same mutants. Similar results were obtained in that whilst 1C1 bound with similar affinity to wild type β_c and the β_c mutants, BION-1 binding was eliminated by the M363A/R364A, E366A and R418A mutants (Table
- 20 I). These results suggest that the epitope recognized by BION-1 is formed, at least in part, by M363 and/or R364, E366 and R418. This is consistent with the disclosure in WO 97/28190 that agents that bind the putative F'-G' loop (of which R418 is part of) will be antagonists of IL-5, GM-CSF and IL-3.

25 BION-1 and IL-3 reciprocally inhibit each other's binding

- To confirm that the epitope recognized by BION-1 was the same or close to the binding site utilized by IL-5, GM-CSF and IL-3, we performed the reverse experiment, in which BION-1 was radio-labelled and increasing concentrations of
- 30 IL-3 used to compete for 125 I-BION-1 binding. The results showed (Figure 7) that IL-3 competed for 125 I-BION-1 binding in a dose-dependent manner emphasizing the close and intimate proximity of BION-1 and IL-3 binding epitopes in β_c .

BION-1 specifically inhibits the function of IL-5, GM-CSF and IL-3 including the stimulation of eosinophil production and activation.

- 35 To ascertain whether the inhibition of IL-5, GM-CSF and IL-3 binding by BION-1 was translated into inhibition of IL-5, GM-CSF and IL-3 stimulation we used the factor dependent TF-1 cell line. This cell line proliferates in the presence

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of either IL-5, GM-CSF, IL-3 or erythropoietin (EPO) (Figure 8). As shown in Figure 8 MoAb BION-1 but not other MoAb anti- β_c nor an IgG₁ control MoAb inhibited the stimulation of TF-1 cell proliferation by IL-5, GM-CSF and IL-3. In contrast, the stimulating ability of erythropoietin was not inhibited showing specificity of BION-1 for the IL-5/GM-CSF/IL-3 receptors system.

Titration experiments showed that BION-1 inhibited cytokine-mediated TF-1 cell proliferation in a dose-dependent manner with an ED₅₀ of about 100-300 nM (Figure 9). Figure 9 also shows that other anti- β_c MoAb were not inhibitory, and that Fab fragments of BION-1 behaved similarly to BION-1 as a whole IgG with virtually overlapping ED₅₀ values.

Since eosinophils are believed to be the major effector cells in asthma and they respond to IL-5, GM-CSF and IL-3, we examined BION-1 for its ability to block eosinophil production, eosinophil survival and eosinophil activation in response to these three cytokines. We found that BION-1 but not MoAb 8E4 inhibited the ability of IL-5, GM-CSF and IL-3 to stimulate the formation of eosinophil colonies from human bone marrow cells (Table II).

Importantly, BION-1 inhibited the pro survival activity of IL-5, IL-3 and GM-CSF on purified peripheral blood human eosinophils. Whilst these cytokines are essential for maintaining eosinophil viability (Figure 10A), blocking of β_c by MoAb BION-1 promotes eosinophil cell death to levels similar to those observed in the absence of cytokines (Figure 10B). Eosinophils can be activated by IL-5, GM-CSF and IL-3 as well as by tumour necrosis factor (TNF- α), a factor that operates through the TNF- α receptor. A sign of eosinophil activation is the upregulation of the CD69 surface antigen, a phenomenon induced by all four cytokines (Figure 11A). Using this activation system we found that BION-1 inhibited the activation of eosinophils by IL-5, GM-CSF and IL-3 (Figure 11B). Other MoAb anti- β_c or IgG₁ controls failed to do so. In addition the blocking effect of BION-1 was found to be specific in that the stimulating activity of TNF- α was not inhibited (Figure 11B).

BION-1 specifically inhibits IL-3 receptor dimerization and activation

In order to define the mechanism of BION-1 antagonism we examined BION-1 for its ability to influence receptor dimerization and activation. We have previously shown that IL-3 or GM-CSF or IL-5 induce dimerization of the respective α

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- chains with β_c , a phenomenon that leads to receptor activation as measured by tyrosine phosphorylation of β_c . This is confirmed here, with Figure 12 showing that in the absence of cytokines antibodies to the α chain (left panel), or β_c (right panel), immunoprecipitate their appropriate antigens (α chain and β_c respectively).
- 5 In the presence of IL-3, dimerization of α and β_c takes place allowing either anti- α chain or anti β_c MoAb to immunoprecipitate both receptor subunits. This is accompanied by tyrosine phosphorylation of β_c (top panel). We show in this figure that pre-incubation of the cells with BION-1 blocks receptor dimerization and tyrosine phosphorylation of β_c . As a control we used the anti β_c MoAb 1C1
- 10 which was unable to prevent receptor dimerization and activation.

BION-1 specifically inhibits chronic myelomonocytic cell growth

- BION-1 is shown to inhibit the activity of one or all of IL-5, IL-3 & GM-CSF mediated effectors of leukaemic cells. In particular BION-1 inhibits growth *in*
- 15 *vitro* of chronic myelomonocytic cells (CMML), whereas a control antibody (1C1) does not (Figure 14). Furthermore, BION-1 inhibits even in the presence of IL-3 whereas the control does not.

Screening and isolation of new inhibitory compounds

- 20 A large range of potential therapeutic compounds that might act as antagonists, or perhaps agonists of IL-3, GM-CSF and IL-5 individually or collectively, can be readily screened. The screening is initially to determine whether the binding of BION-1 or a fragment thereof to β_c receptor or fragment is inhibited. The nature of these inhibitory compounds will not be limited, and the methods used for a
- 25 binding assay can be any one of the many techniques known to those skilled in the art. Such methods may include affinity selection chromatography, ultrafiltration assays, the scintillation proximity assay, interfacial optical techniques, the quartz crystal microbalance, the jet ring cell, interferometric assays using porous silicon to immobilise the receptor. Reference to such techniques can be found in
- 30 Woodbury *et al* 1999, which reference is incorporated herein in its entirety.

- The range of therapeutic compounds may include peptides, oligonucleotides, or other small organic or inorganic molecules. Figure 13 shows the results of screening 7-mer and 12-mer peptide libraries using soluble β_c domain 4 supported
- 35 on ELISA plates.

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DEPOSIT OF CELL LINE

The cell line BION-1 was deposited on the April 29th, 1998 in the American Type Culture Collection (ATCC) at 101801 University Boulevard, Manassas, Virginia, United States of America and has been designated ATCC HB-12525.

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Table I Epitope mapping of Bion-1. Binding affinities of MoAb Bion-1 tested on COS cells transfected with wild type β_c or mutants of β_c

5	β_c wild type:	Bion-1 KD	ICI KD
		49.3*	4.4
	β_c mutated in the B'-C' loop:		
	M163A/R364A	0†3.8	
	Y165A	69.41.5	
10	E356A	02.4	
	H167A	27.02.8	
	I358A	21.72.4	
	D169A/H370A	32.43.6	
	β_c mutated in the F'-G' loop:		
15	R418A	0	1.8
	T419A	23.3	3.2
	G420A	53.0	1.5
	Y421A	38.9	2.3
	* K_D in nM		
20	0† = not detectable binding		

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Table II Inhibition of IL-5, GM-CSF and IL-3 mediated eosinophil colony formation by BION-1

5	Medium	[MoAb 8E4] (100 μ M)		[MoAb BION-1] (μ M)		
		0.1	1	10	100	
	IL-5 (1nM)	13 \pm 4*	15 \pm 3	13 \pm 4	8 \pm 2	2 \pm 2
	GM-CSF (2nM)	9 \pm 4	18 \pm 4	20 \pm 4	13 \pm 4	2 \pm 2
	IL-3 (2nM)	4 \pm 2	8 \pm 1	8 \pm 2	4 \pm 1	1 \pm 1
10	NONE	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	2 \pm 0

*Number of day 14 eosinophil colonies per 10^5 seeded bone marrow cells. Values shown are the mean from triplicate determination \pm SEM.

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INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>23</u> line <u>1-4</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 10801 University Blvd Manassas, VA 20110-2209 United States of America	
Date of deposit 29 April 1998	Accession Number HB-12525
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only	
<input checked="" type="checkbox"/> This sheet was received with the international application	
Authorized officer (Mrs) Anne HAMMETT (02) 6283 2225	
For International Bureau use only	
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Authorized officer	

CLAIMS

1. A method of isolating a monoclonal antibody capable of inhibiting any one of IL-3, GM-CSF and IL-5 binding to the common receptor β_c , or a receptor analogous to β_c , said method including the steps of:
 - 5 immunising an animal with a cytokine receptor or portion of a cytokine containing at least the extracellular domain 4 or analogous domain in the analogous common receptor or part thereof,
 - isolating antibody producing cells from said animal,
 - fusing antibody producing cells with a myeloma cell line, and
 - 10 screening for a cell line that produces the monoclonal antibody of capable of inhibiting any one of IL-3, GM-CSF and IL-5 binding to the common receptor β_c , or a receptor analogous to β_c .
2. A method as in claim 1 wherein the immunisation involves introducing a
 - 15 cDNA clone of a portion of or all of the common receptor including the extracellular domain 4 or analogous domain in the analogous common receptor or part thereof, into a cell and proliferating said cells to form a recombinant cell line, inoculating an animal with said recombinant cell line, isolating antibody producing cells from said animal and fusing the antibody producing cell line with a myeloma
 - 20 cell line to form a hybridoma cell line, screening for a hybridoma cell line that produces an antibody that binds to the recombinant cell line but not to the parent, and then testing for inhibition against all three cytokines.
3. A method as in claim 2 wherein the cell into which the cDNA clone is
 - 25 introduced is mammalian.
4. A method as in claim 3 wherein the mammalian cell line is a COS cell.
5. A method as in claim 2 wherein the cDNA encodes a full or partial portion
 - 30 of domain 4 when it is in a configuration where the F'-G' loop and/or the B'-C' loop is in its native shape.
6. A method as in claim 2 wherein the domain 4 of β_c or equivalent domain in
 - 35 other cytokine receptors is expressed in isolation in a microbial host and used to immunise animals for developing monoclonal antibodies.

30

7. A method as in claim 2 wherein the analogous receptor is any one of the cytokine superfamily receptors from the group including β_c , LIFR, gp130, IL-2R β , IL-4R/IL-13R, IL-2R γ , IL-3R α , EPOR, TPOR and OBR.
- 5 8. A method as in claim 2 wherein the method is used to isolate a monoclonal antibody that inhibits binding of all of the said receptors to a common receptor.
9. A method as in claim 7 wherein the common receptor is selected from the group of receptors acting for more than one cytokine including but not limited to
10 gp130, LIFR, IL2R β /IL2R α , IL-4R/IL-13R and β_c .
10. A monoclonal antibody, or fragments thereof capable of inhibiting the binding of the cytokines IL-3, GM-CSF and IL-5 to the β_c receptor.
- 15 11. A monoclonal antibody as in claim 10 wherein the monoclonal antibody or fragment thereof binds to at least the F'-G' loop of domain 4 of the β_c subunit.
12. A monoclonal antibody as in claim 10 wherein the monoclonal antibody or fragment thereof binds to at least the B'-C' loop of domain 4 of the β_c subunit.
20
13. A monoclonal antibody as in claim 10 wherein the monoclonal antibody or fragments thereof binds to both the F'-G' as well as the B'C' loop of domain 4 of the β_c .
- 25 14. A monoclonal antibody as in claim 10 wherein the monoclonal antibody inhibits β_c receptor dimerisation.
15. A monoclonal antibody as in claim 10 wherein nucleic acid encoding the variable region of the monoclonal antibody is recombined with nucleic acid
30 encoding non-variable regions of human origin in an expression vector.
16. A monoclonal antibody as in claim 10 wherein the inhibition leads to blocking of at least one function of all three cytokines.
- 35 17. A monoclonal antibody as in claim 10 wherein the activity leads to inhibition of stimulation of effector cell activation or survival.

31

18. A monoclonal antibody as in claim 17 wherein the antibody or fragment thereof is used for treatment of asthma and leads to inhibition of IL-5, IL-3 & GM-CSF mediated eosinophil activation.
- 5 19. A monoclonal antibody as in claim 17 wherein the antibody or fragment thereof is used for treatment of asthma and leads to inhibition of IL-5, IL-3 & GM-CSF mediated eosinophil survival.
- 10 20. A monoclonal antibody as in claim 17 wherein the effector cell is selected from the list including leukaemic cells, endothelial cells, breast cancer cells, prostate cancer cells, small cell lung carcinoma cells, colon cancer cells, macrophages in chronic inflammation, and dendritic cells for immunosuppression.
- 15 21. A monoclonal antibody as in claim 17 wherein the monoclonal antibody is the antibody produced by the hybridoma cell line BION-1 (ATCC HB-12525).
22. A hybridoma cell line capable of producing the monoclonal antibody of claim 10.
- 20 23. A hybridoma cell line as in claim 22 wherein the hybridoma cell line is BION-1 (ATCC HB-12525).
- 25 24. A method of isolating an inhibitor capable of competitively inhibiting the binding of BION-1 or the binding of an agent capable of inhibiting BION-1 binding, to the β_c subunit, the method including the steps of contacting BION-1 or fragment thereof with the β_c subunit or fragment thereof as well as a candidate inhibitory compound,
and measuring the degree of binding.
- 30 25. A method as in claim 24 wherein a reporting means is provided to facilitate the detection of binding of BION1 or fragment thereof with β_c subunit or fragment thereof.
- 35 26. A method as in claim 24 wherein the inhibitor is a peptide or a nucleotide molecule.
27. An inhibitor isolated by the method of claim 24.

32

28. A cytokine inhibitor that simultaneously blocks the binding of β_c by IL-3, GM-CSF, and IL-5.

5 29. An inhibitor of leukaemic cell proliferation wherein the inhibitor inhibits binding of IL-3, GM-CSF and IL-5 with β_c subunit or fragment thereof.

30. An inhibitor as in claim 29 wherein the proliferation of the cell is cytokine dependent.

10

31. An inhibitor as in claim 29 wherein the inhibitor is BION-1 or an agent capable of inhibiting BION-1 binding with β_c subunit or fragment thereof.

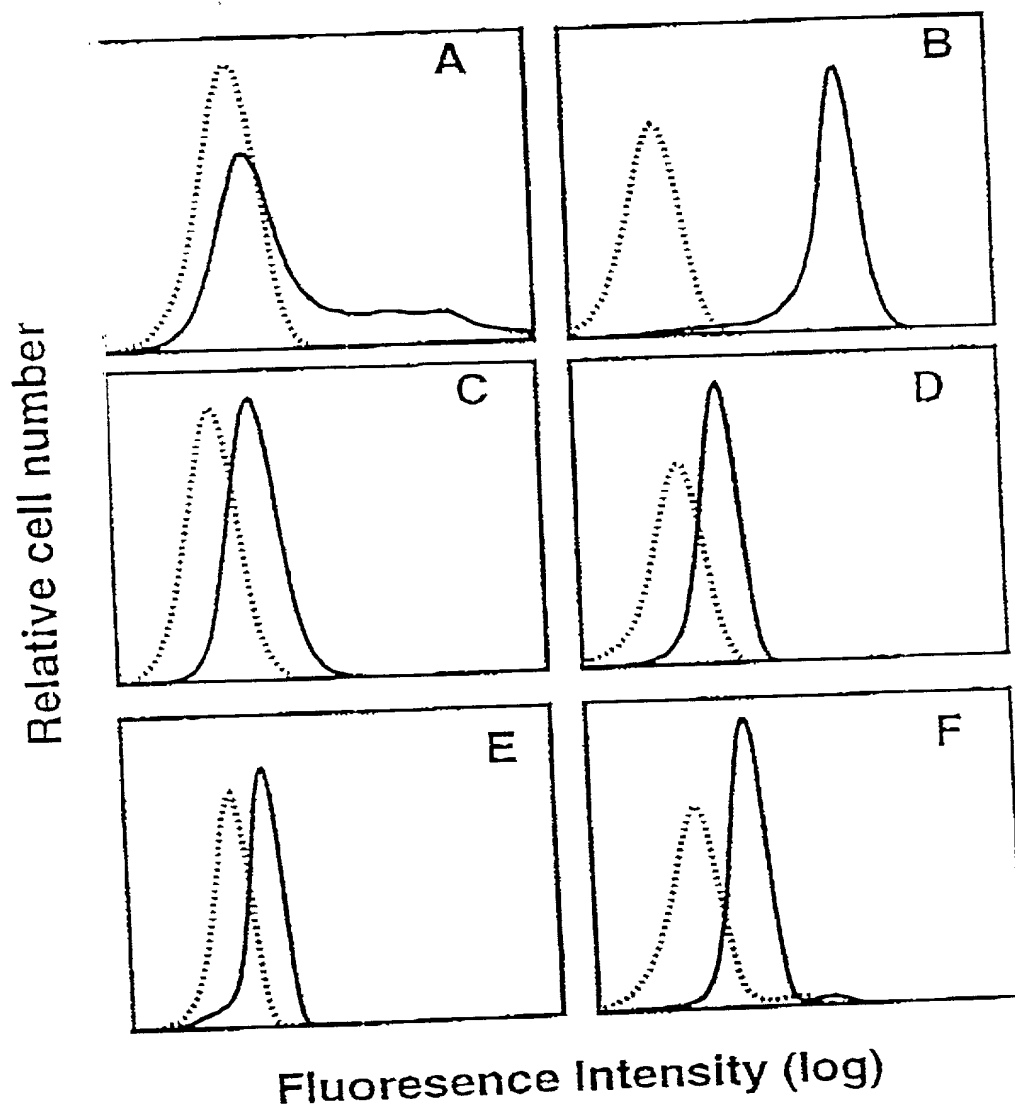


FIGURE 1

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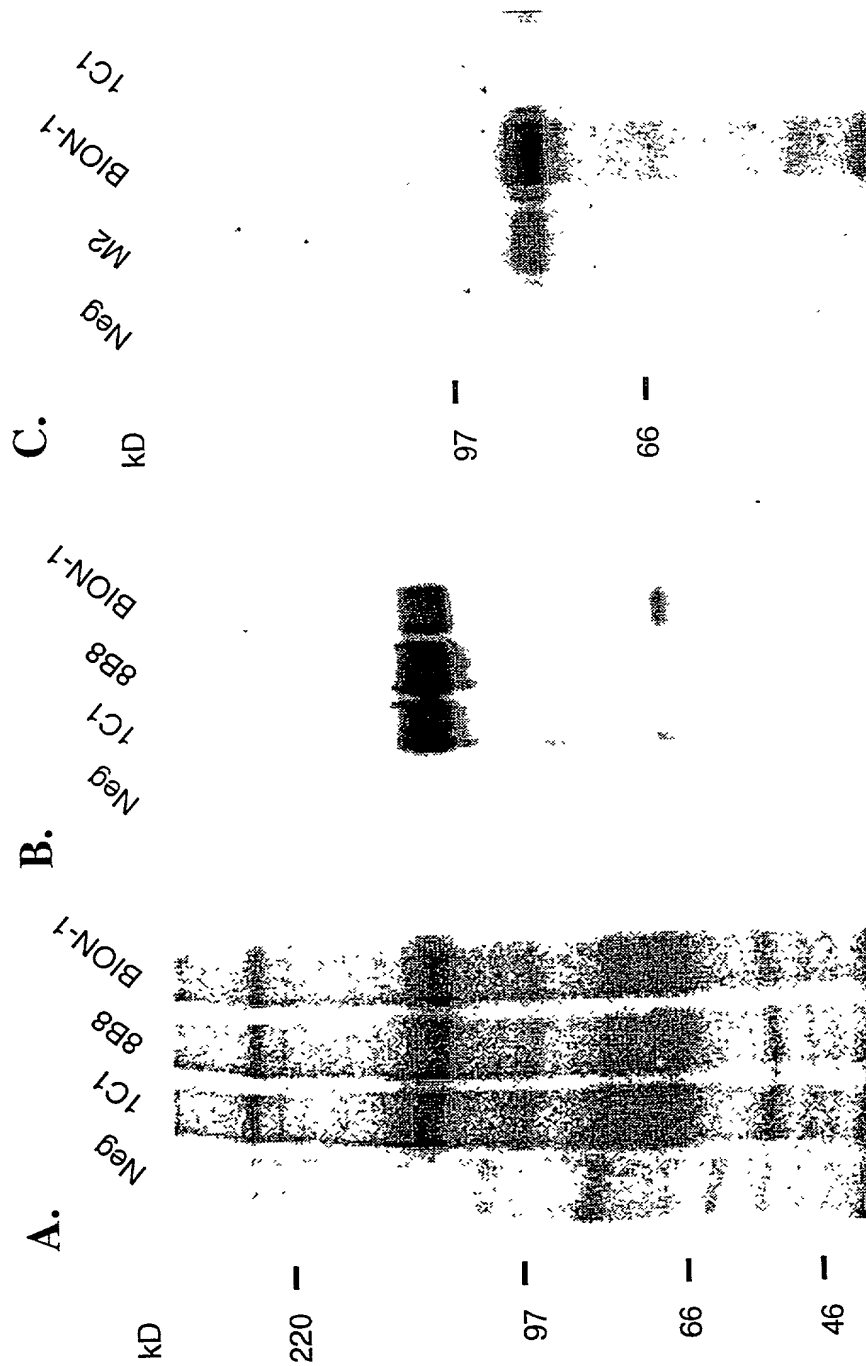


FIGURE 2

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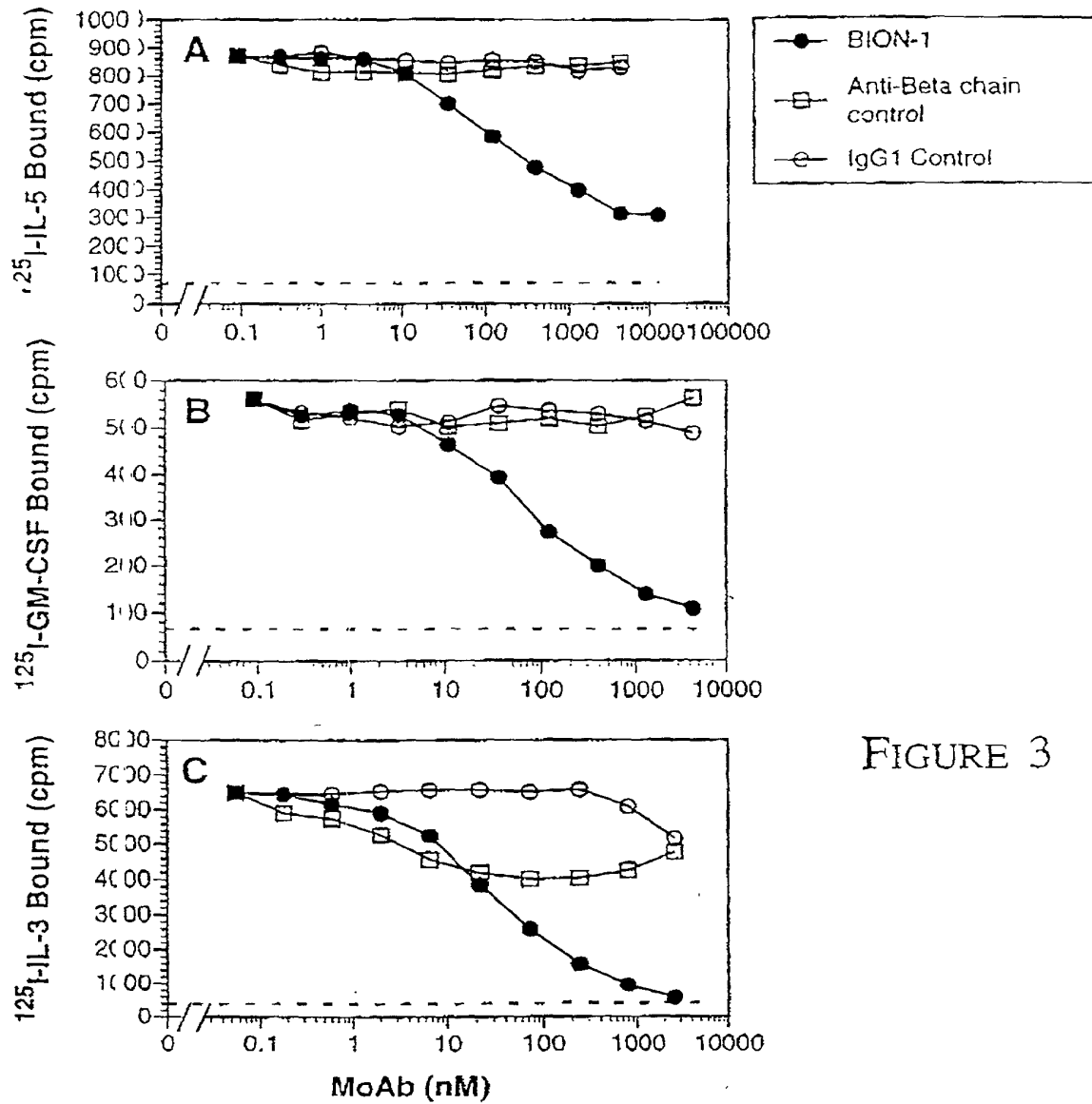


FIGURE 3

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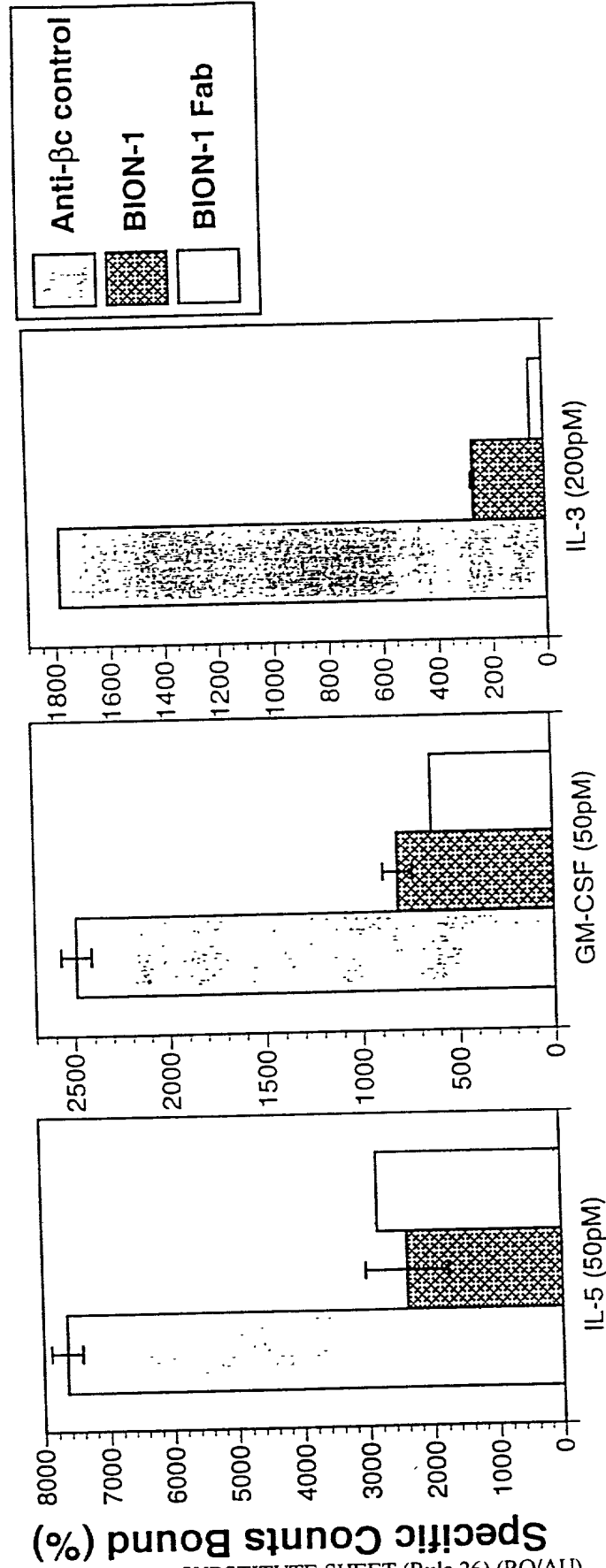


FIGURE 4
 ^{125}I -Cytokine

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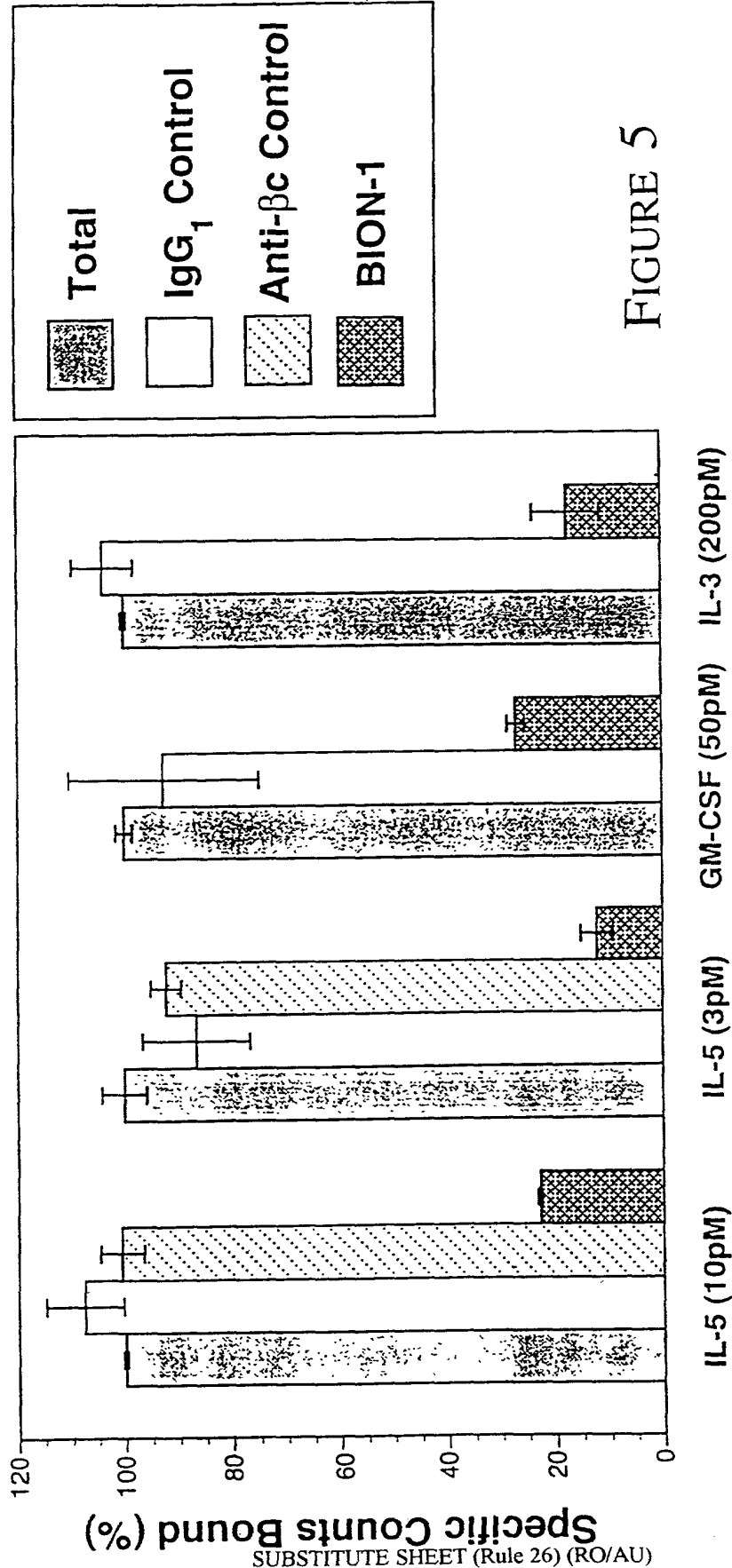


FIGURE 5

¹²⁵I-Cytokines

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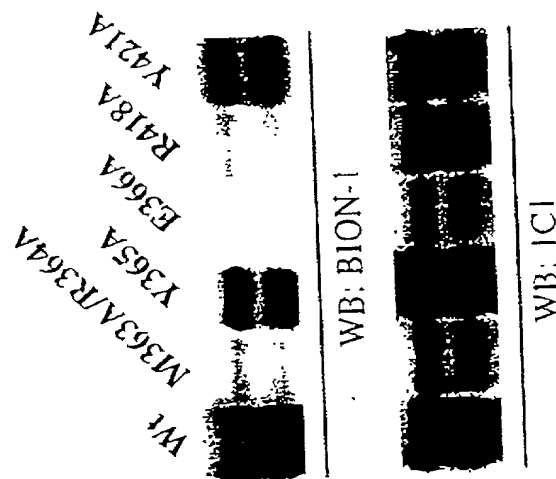


FIGURE 6

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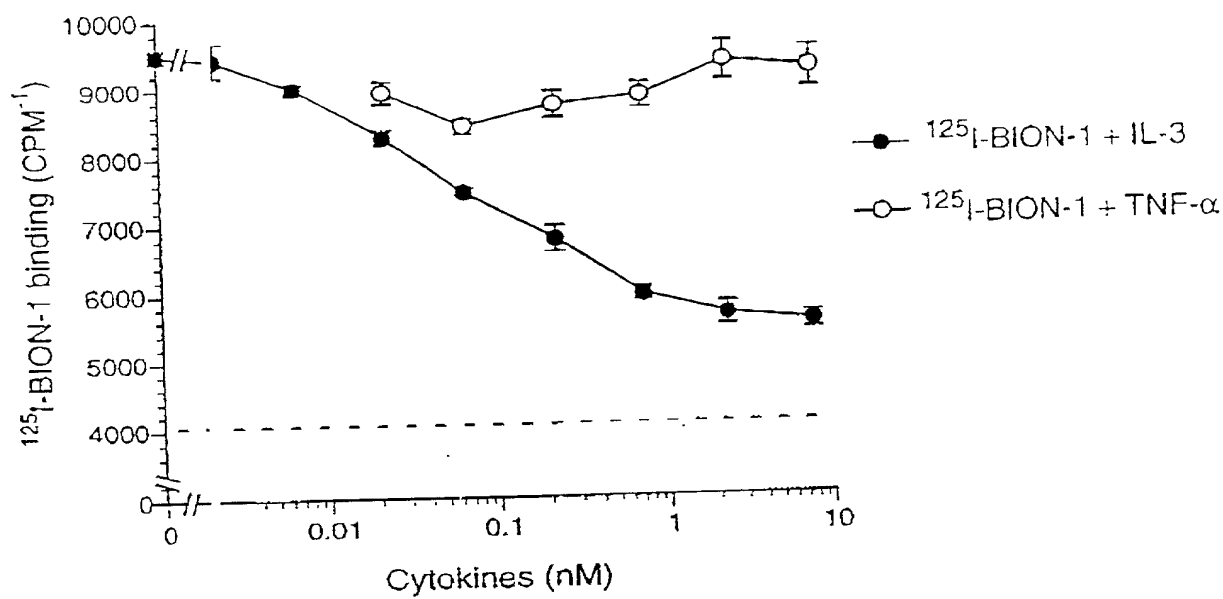


FIGURE 7

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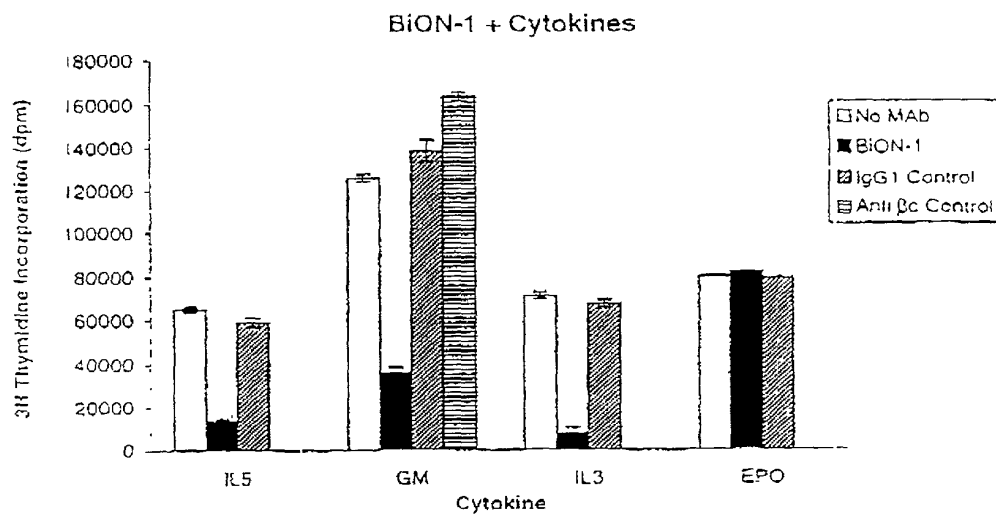
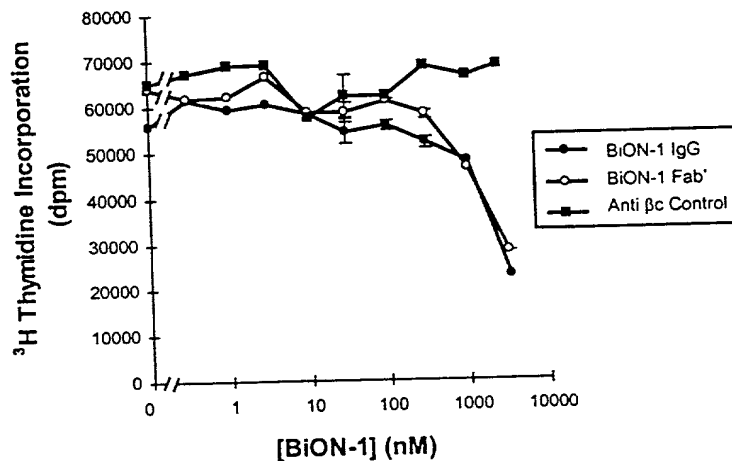


FIGURE 8

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BiON-1 + IL5 (0.3ng/ml)



BiON-1 + GM-CSF (0.03ng/ml)

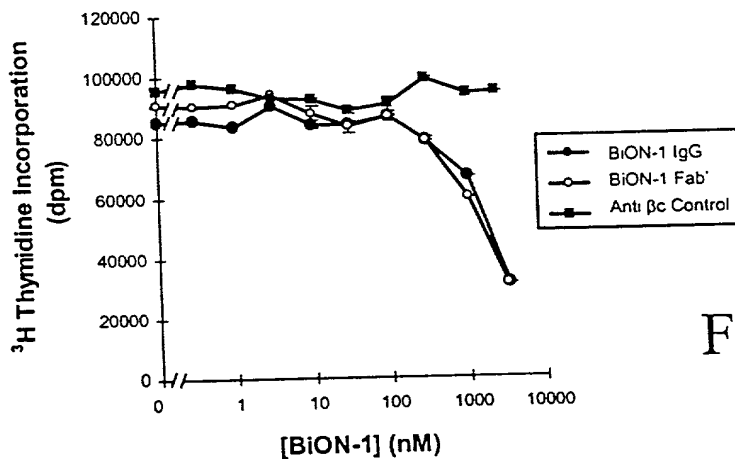
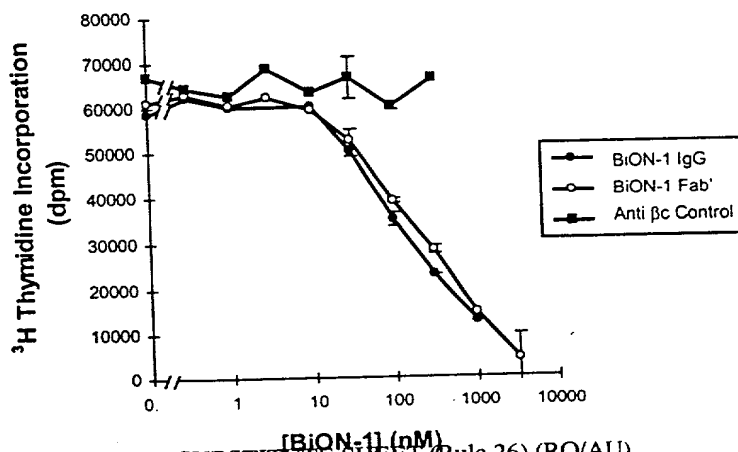


Figure 9

BiON-1 + IL3 (0.3ng/ml)



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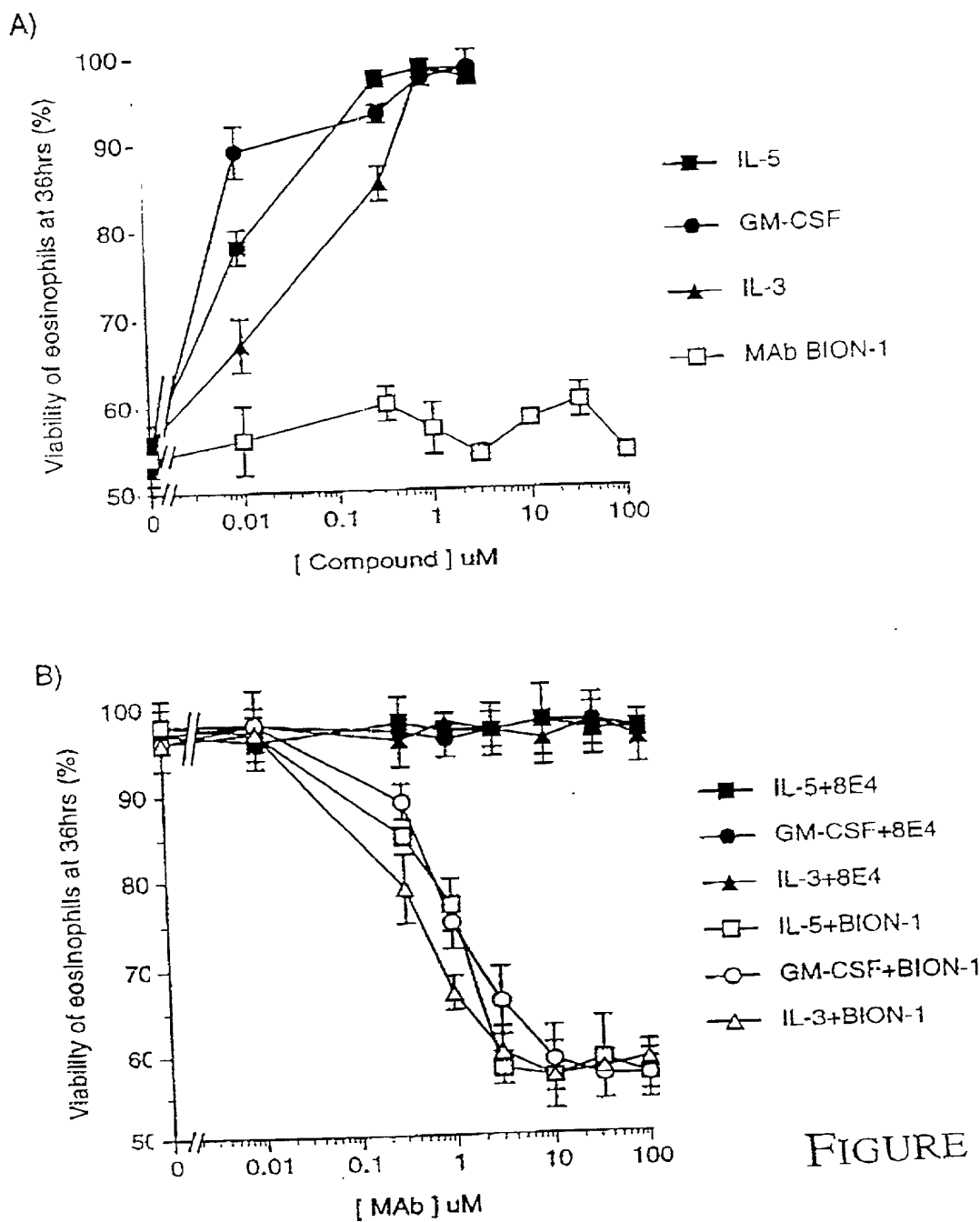


FIGURE 10

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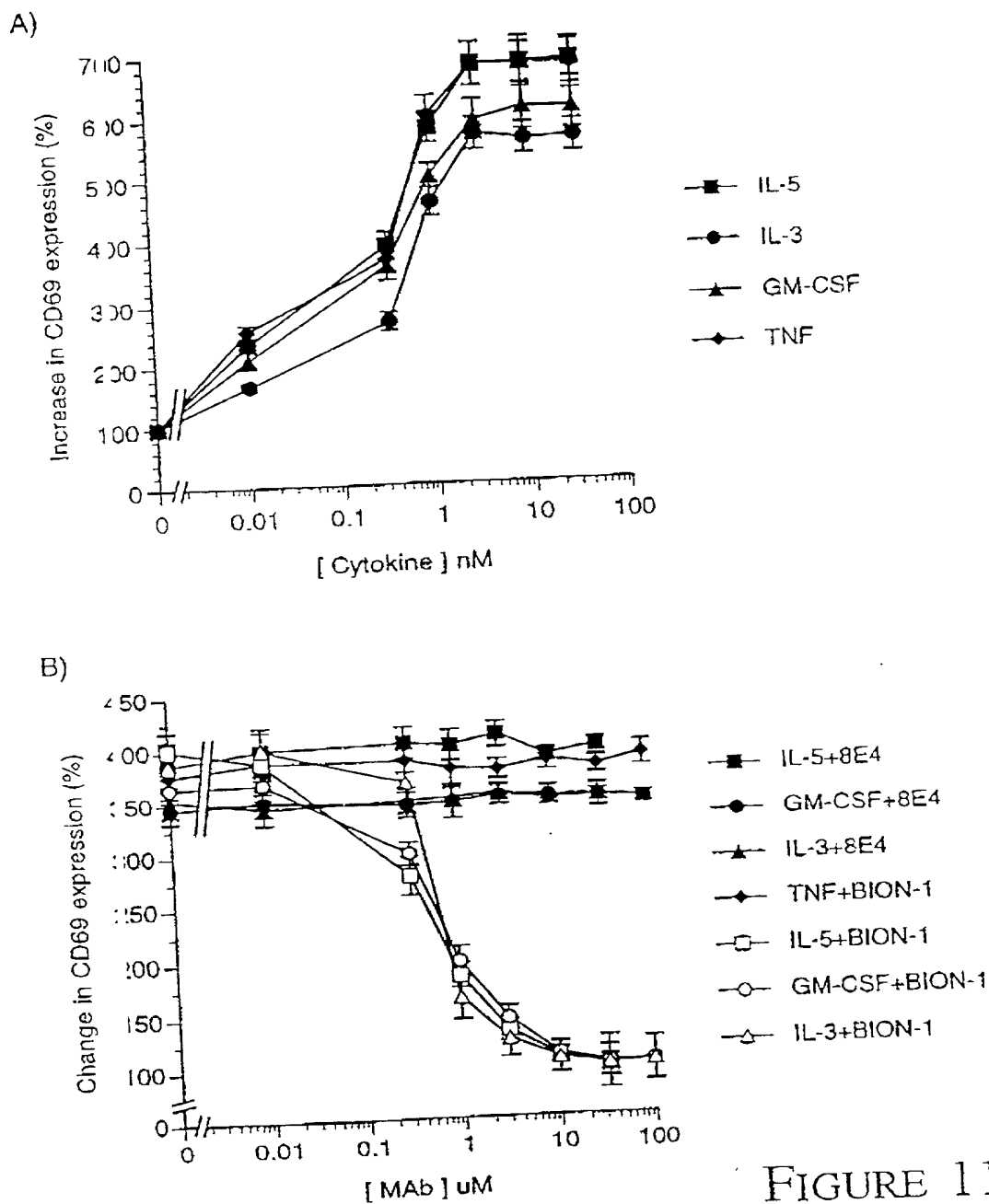


FIGURE 11

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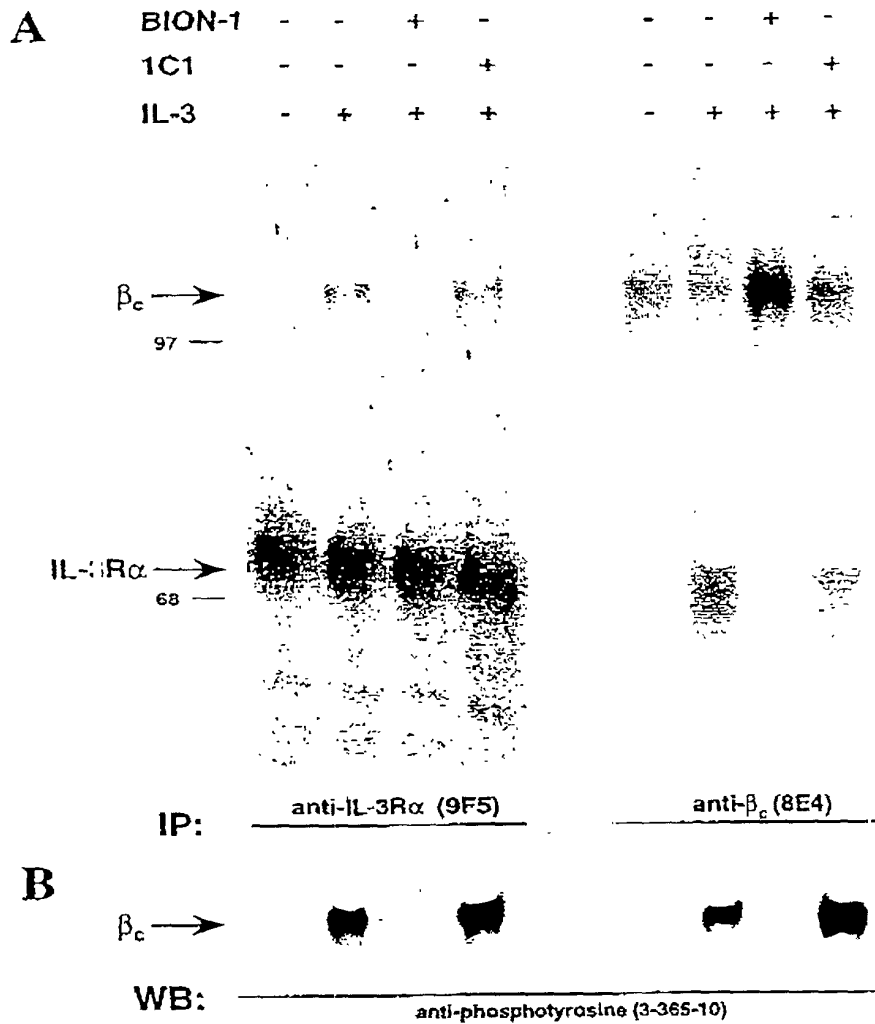


FIGURE 12

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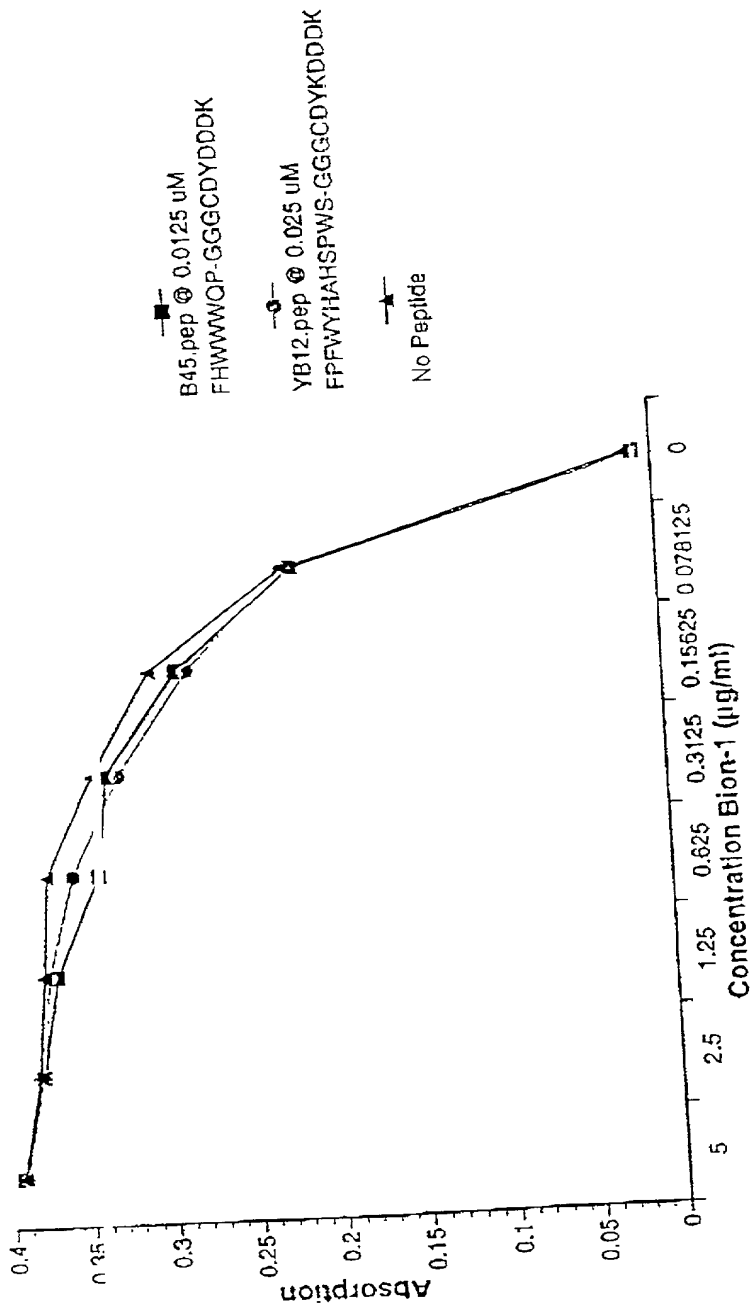


FIGURE 13

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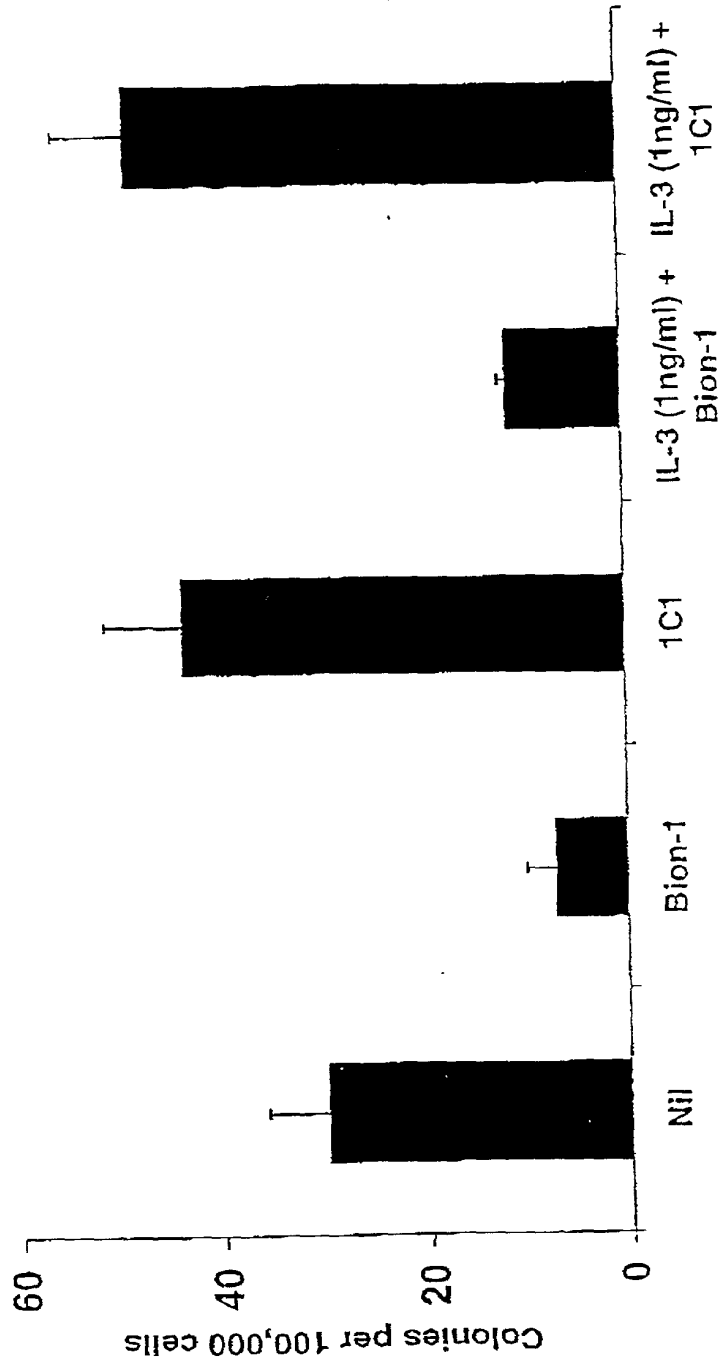


FIGURE 14

**COMBINED DECLARATION AND POWER OF ATTORNEY FOR UNITED STATES
PATENT APPLICATION**

As a below named inventor, we hereby declare that:

My residence, post office address and citizenship are the same as stated below next to my name.

We believe we are an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:-

**MONOCLONAL ANTIBODY INHIBITOR OF GM-CSF, IL-3, IL-5 AND OTHER
CYTOKINES, AND USES THEREOF**

the specification of which



was filed as PCT International Application No. PCT/AU99/00659
on 13th August 1999

We hereby state that we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information known to us to be material to the examination of this application in accordance with Title 37, Code of Federal Regulations, S1.56(a).

We hereby claim foreign priority benefits under Title 35, United States Code, S119 of any foreign application for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the applicant on which priority is claimed.

Number	Country	Filing Date (d/m/y)	Priority Claimed
PP5251	Australia	13th August 1998	<input checked="" type="checkbox"/> yes <input type="checkbox"/> no

We hereby claim the benefit under Title 35, United States Code, S120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, S112, we acknowledge the duty to disclose all information known to us to be material to patentability as defined in Title 37, Code of

Federal Regulation, S1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

Application Serial No. Filing Date (d/m/y) Status (Patented, Pending, Abandoned)

We hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

3

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708 Third Avenue 14th Floor
NEW YORK NEW YORK 10017-4104
UNITED STATES OF AMERICA

We hereby declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Date

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6/2/01
Date

R. D'Andrea
Richard D'Andrea